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RESEARCH PAPER

Analysis of the contribution of I_{to} to repolarization in canine ventricular myocardium

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BACKGROUND AND PURPOSE

The contribution of the transient outward potassium current (I_{to}) to ventricular repolarization is controversial as it depends on the experimental conditions, the region of myocardium and the species studied. The aim of the present study was therefore to characterize I_{to} and estimate its contribution to repolarization reserve in canine ventricular myocardium.

EXPERIMENTAL APPROACH

Ion currents were recorded using conventional whole-cell voltage clamp and action potential voltage clamp techniques in canine isolated ventricular cells. Action potentials were recorded from canine ventricular preparations using microelectrodes. The contribution of I_{to} to repolarization was studied using 100 μM chromanol 293B in the presence of 0.5 μM HMR 1556, which fully blocks I_{Ks}.

KEY RESULTS

The high concentration of chromanol 293B used effectively suppressed I_{to} without affecting other repolarizing K^+ currents (I_{K1}, I_{Kr}, I_p). Action potential clamp experiments revealed a slowly inactivating and a 'late' chromanol-sensitive current component occurring during the action potential plateau. Action potentials were significantly lengthened by chromanol 293B in the presence of HMR 1556. This lengthening effect induced by I_{to} inhibition was found to be inversely rate-dependent. It was significantly augmented after additional attenuation of repolarization reserve by 0.1 μM dofetilide and this caused the occurrence of early afterdepolarizations. The results were confirmed by computer simulation.

CONCLUSIONS AND IMPLICATIONS

The results indicate that I_{to} is involved in regulating repolarization in canine ventricular myocardium and that it contributes significantly to the repolarization reserve. Therefore, blockade of I_{to} may enhance the risk of the development of pro-arrhythmias.

Abbreviations

APD, action potential duration; APD_{50} and APD_{90}, action potential durations at 50% and 90% of repolarization; DPP, dipeptidyl-aminopeptidase-like protein; EAD, early afterdepolarization; I_{Ca}, L-type calcium current; I_{K1}, inward rectifier potassium current; I_{Kr}, rapid component of the delayed rectifier potassium current; I_{Ks}, slow component of the delayed rectifier potassium current; I_{Na}, sodium current; I_p, plateau current; I_{to}, transient outward potassium current; KChIP, Kv channel interacting protein; LQTS, long QT syndrome

Introduction

Repolarization in mammalian ventricular muscle is controlled by a fine balance of several inward and outward transmembrane ionic currents and electrogenic pump mechanisms, and as such, it seems rather complex. This complexity is mainly due to dynamic interactions of these currents – many of them depending on both voltage and

intracellular Ca^{2+} – during the course of the action potential. Therefore, the relative contribution of an individual current to the repolarization process is difficult to assess. One approach is to apply selective ion channel blocking compounds, in this case the resulting changes in the action potential waveform provide the information. The pharmacological approach can also provide useful information; in this case drugs are used to selectively block specific currents, such as the rapid (I_{Kr}) and slow (I_{Ks}) delayed rectifier, the inward rectifier (I_{K1}) potassium currents, the L-type calcium (I_{Ca}) and sodium (I_{Na}) currents. However, this method has limitations as some transmembrane currents cannot be blocked selectively by drugs.

The calcium-independent transient outward current (I_{to}) is a transmembrane current activating and inactivating rapidly during depolarization to voltages more positive than –20 mV. From inactivation the current can recover either rapidly or slowly, depending on the type of the pore forming alpha subunit (Kv4.3 and Kv1.4 respectively). The magnitude and kinetics of I_{to} are also influenced by the expression of various beta subunits (KChIP2 or DPP) (Patel and Campbell, 2005; Radicke *et al.*, 2005). The I_{to} is also largely heterogeneous within the ventricular wall and shows marked interspecies variability (Antzelevitch *et al.*, 1991; Wettwer *et al.*, 1994; Näbauer *et al.*, 1996; Li *et al.*, 1998). It is absent in the guinea-pig and pig (Sipido *et al.*, 1995; Li *et al.*, 2003), but is prominent in rabbit, dog, ferret and human ventricular muscle (Hiraoka and Kawano, 1989; Tseng and Hoffman, 1989; Campbell *et al.*, 1993; Näbauer *et al.*, 1993; Sipido *et al.*, 1993). In rabbits I_{to} is mediated mostly by Kv1.4 channels as it has very slow recovery kinetics (Hiraoka and Kawano, 1989; Patel and Campbell, 2005); thus, the resultant current is largely inactivated at fast rates. In dogs and humans I_{to} recovers rapidly from inactivation, so the current can be active at a wide range of pacing frequencies. It is generally accepted that I_{to} – due to its fast activation and inactivation upon depolarization – plays an important role only at the beginning of the action potential, contributing to phase 1 repolarization and the notch. It has also been suggested that changes in I_{to} may alter the plateau voltage, and consequently, it can indirectly influence the activation and deactivation of other currents active during the plateau phase (Priebe and Beuckelmann, 1998; Sun and Wang, 2005). Also, the I_{to} was suggested to participate in the excitation–contraction coupling and Brugada syndrome (Sah *et al.*, 2003; Antzelevitch, 2006). However, the contribution of I_{to} to the overall duration of the action potential, and consequently to the effective refractory period has not been elucidated and is still controversial.

The major problem of establishing the role of I_{to} in repolarization is the lack of a selective blocker, which would allow us to study its contribution to cardiac repolarization directly. The commonly used I_{to} blocker 4-aminopyridine has been reported to alter several other important transmembrane ion currents (Ridley *et al.*, 2003); therefore, it cannot be used as a selective I_{to} inhibitor. Phrixotoxin and heteropodatoxin have been reported to block Kv4 channels in cell lines (Sanguinetti *et al.*, 1997; Diochot *et al.*, 1999) but our preliminary experiments (not shown) did not reveal an effect of these toxins on I_{to} in dog ventricular myocytes. In an earlier study from our laboratory we demonstrated that block of I_{Ks} by 10 μM chromanol 293B or 100 nM L-735 821 failed to change significantly action potential duration in dog and human ventricular muscle at normal length of repolarization and in the absence of symphathetic stimulation (Varró *et al.*, 2000; Jost *et al.*, 2005). High concentrations of chromanol 293B, a drug used to block I_{Ks} selectively at low (micromolar) concentrations, has also been shown to effectively suppress I_{to} as well (Bosch *et al.*, 1998; Sun *et al.*, 2001). Taking advantage of this observation, in the present study we applied high concentrations of chromanol 293B in canine subepicardial muscle preparations and ventricular myocytes – by using the conventional microelectrode technique, patch clamp and action potential voltage clamp techniques – to obtain direct information as to how inhibition of I_{to} may alter ventricular repolarization and how these possible changes can be related to the properties of I_{to}. Furthermore we estimated the contribution of I_{to} to repolarization reserve and also performed a computer simulation study.

Methods

All experiments were carried out in compliance with the *Guide for the Care and Use of Laboratory Animals* (USA NIH publication NO 85-23, revised 1985). The protocols were approved by the Review Board of the Committee on Animal Research of the Animal Health and Animal Welfare Directorate, Hungary (15.1/01031/006/2008).

Conventional microelectrode technique

Adult mongrel dogs (8–14 kg) of either sex were used. Following anaesthesia (sodium pentobarbital, 30 $mg \cdot kg^{-1}$ i.v.), the heart was rapidly removed through right lateral thoracotomy. The hearts were immediately rinsed in oxygenated modified Locke solution containing (in mM): NaCl 120, KCl 4, $CaCl_2$ 1.0, $MgCl_2$ 1, $NaHCO_3$ 22 and glucose 11. The pH of this solution was set between 7.35 and 7.4 when saturated with the mixture of 95% O2 and 5% CO2 at 37°C. Subepicardial muscle preparations obtained from the right ventricle were individually mounted in a tissue chamber having a volume of 50 mL. These preparations were stimulated (HSE stimulator, type 215/II, Hugo Sachs Elektronik, March – Hugstetten, Germany) through a pair of platinum electrodes in contact with the preparation using rectangular current pulses of 2 ms duration. These stimuli were delivered at a constant cycle length of 1 s for at least 60 min allowing the preparation to equilibrate before the measurements were initiated. Transmembrane potentials were recorded using conventional glass microelectrodes, filled with 3 M KCl and having tip resistances of 5–20 MΩ, connected to the input of a high-impedance electrometer (Experimetria, type 309, Budapest, Hungary) which was coupled to a dual beam oscilloscope. The maximum diastolic potential, action potential amplitude and action potential duration measured at 50% and 90% of repolarization (APD_{50} and APD_{90} respectively) were determined off-line using a home-made software running on an IBM compatible computer equipped with an ADA 3300 analogue-to-digital data acquisition board (Real Time Devices Inc., State Collage, PA, USA) having a maximum sampling frequency of 40 kHz. Attempts were made to maintain the same impalement throughout each experiment. If, however,

an impalement became dislodged, adjustment was attempted, and if the action potential characteristics of the re-established impalement deviated by less than 5% from the previous measurement, the experiment was continued.

Whole-cell patch clamp

Ventricular myocytes were enzymatically dissociated from dog hearts using the segment perfusion technique as described earlier in detail (Varró *et al.*, 2000). One drop of cell suspension was placed in a transparent recording chamber mounted on the stage of an inverted microscope. The myocytes were allowed to settle and adhere to the bottom for at least 5 min before superfusion was initiated. Only rod shaped cells with clear cross-striations were used. Cells were superfused with HEPES-buffered Tyrode solution containing (in mM): NaCl 144, NaH_2PO_4 0.33, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and HEPES 5.0. The pH was set to 7.4 and the temperature to 37°C.

Patch-clamp micropipettes were fabricated from borosilicate glass capillaries (Clark, Reading, UK) using a micropipette puller (Flaming/Brown, type P-97, Sutter Co, Novato, CA, USA). These electrodes had resistances between 1.5 and 2.5 MΩ when filled with pipette solution containing (in mM): K-aspartate 100, KCl 40, ATP 5, $MgCl_2$ 5, EGTA 4, $CaCl_2$ 1.5 and HEPES 10. The pH of this solution was adjusted to 7.2 by KOH. When measuring potassium currents, 1 μM nisoldipine (gift from Bayer AG, Leverkusen, Germany) was added to the external solution to eliminate L-type Ca^{2+} current (I_{Ca}). The slow component of the delayed rectifier potassium current (I_{Ks}) was inhibited by using the selective I_{Ks} blocker HMR 1556 (0.5 μM). In some experiments the rapid component of the delayed rectifier potassium current (I_{Kr}) was blocked by 0.1 μM dofetilide. Membrane currents were recorded with Axopatch-1D and 200B patch-clamp amplifiers (Axon Instruments, Union City, CA, USA) using the whole-cell configuration of the patch-clamp technique. After a high-resistance (1–10 GΩ) seal had been established by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by suction or application of short electrical pulses. The series resistance typically ranged from 4 to 8 MΩ before compensation (50–80%). Experiments were discarded, when the series resistance was high or substantially increased during the measurement. Membrane currents were digitized after low-pass filtering at 1 kHz using a 333 kHz analogue-to-digital converter (Digidata 1200, Axon Instruments) under software control (pClamp 8.0 Axon Instruments). The same software was used for off-line analysis.

Action potential voltage clamp

After formation of whole-cell configuration, action potentials were recorded in current clamp mode from the myocytes superfused with Tyrode solution, that also contained 0.5 μM HMR 1556 in order to block I_{Ks}. I_{Ks} blockade by HMR 1556 had little effect on action potential configuration (Varró *et al.*, 2000). The cells were continuously paced through the recording electrode at a steady stimulation frequency of 1 Hz. Ten subsequent action potentials were recorded from each cell, which were digitized and averaged. This averaged signal was delivered to the same cell at an identical frequency as the command voltage, after the amplifier had been switched to voltage clamp mode. The current trace obtained under these conditions was a horizontal line positioned at the zero level except for the very short segment corresponding to the action potential upstroke. Chromanol 293B was applied at a concentration of 100 μM in the continuous presence of HMR 1556; this produced an inward compensatory current, the inversion of this current (C) was then defined as I_{to}.

In another set of experiments in whole-cell voltage clamp mode an action potential waveform, which was previously recorded from a dog left ventricular epicardial slice with a sharp microelectrode by conventional microelectrode technique, was delivered as a command potential with a frequency of 0.2 Hz. After current traces had been recorded under control conditions 100 μM chromanol 293B was applied. Chromanol 293B sensitive current was defined as I_{to} by subtracting the post-drug curve from the pre-drug one. I_{Ks}, I_{Kr} and I_{Ca} currents were pharmacologically blocked throughout the measurements.

Statistics

Results were compared using Student's *t*-tests for paired and unpaired data as appropriate. Differences were considered significant when *P* was less than 0.05. Data are expressed as arithmetic mean ± SEM values.

Computer simulations

Canine epicardial steady-state action potentials and the underlying ionic currents were simulated using the recently published Decker *et al.* (2009) model. Simulations mimicking the action potential experiments were performed using both the original model and a modified version in which I_{to} was reformulated.

The Decker *et al.* (2009) dynamic model for the canine epicardial action potential contains mathematical descriptions for 16 transmembrane currents (corresponding to different channels, pumps and exchangers) and intracellular calcium handling processes. In some of our simulations, we have only modified the formulation of the 4-aminopyridine-sensitive transient outward current (I_{to1}) as follows.

As in the original Decker *et al.* (2009) model, I_{to1} is formulated as:

$$I_{to1} = G_{to1} a^3 i_f i_s (V - E_k)$$

where G_{to1} is the maximum conductance, a is the activation gate, if and is are the fast and slow inactivation gates, respectively, V is membrane potential, and E_K is the Nernst potential for K^+ ions.

The steady-state curves for the gates were reformulated using the data shown in Figure 1E, yielding:

$$a_\infty = \frac{1}{1+\exp\left(-\frac{V+14.65}{19.78}\right)} \qquad i_{f,\infty} = i_{s,\infty} = \frac{1}{1+\exp\left(\frac{V+40.08}{8.496}\right)}$$

The time constants for the inactivation gates were also reformulated to fit the data shown in Figure 1D, as well as unpublished data from our group, yielding:

$$\alpha_{if} = \frac{1}{150\left[1+\exp\left(\frac{V+58}{5}\right)\right]} \qquad \beta_{if} = \frac{1}{5\left[1+\exp\left(-\frac{V+19}{9}\right)\right]}$$

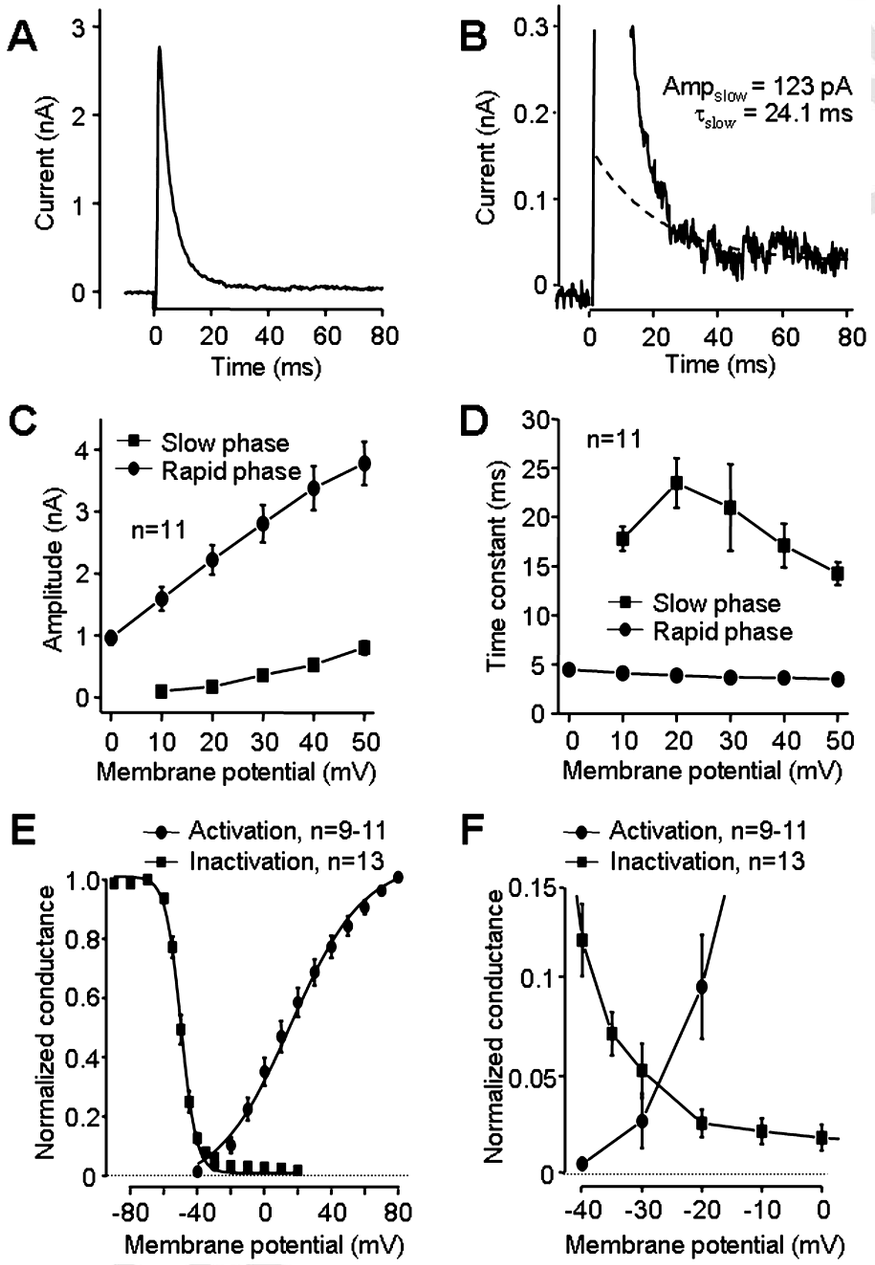

Figure 1

(A) Representative I_{to} trace recorded at +20 mV from a canine ventricular myocyte. The ordinate was enlarged tenfold in (B) where the dashed line indicates the slowly inactivating component of the current generated by simulation using the estimated amplitude and the time constant values. (C, D) The decay of I_{to} was fitted as a sum of two exponentials. The estimated amplitudes (C) and time constants (D) are presented as a function of the membrane potential. (E, F) Steady-state activation and inactivation curves obtained for I_{to} in canine ventricular myocytes. The overlapping region is enlarged in (F). Data are expressed as mean ± SEM. I_{to}, transient outward potassium current; *n*, number of measurements.

$$\alpha_{is} = \frac{1}{800\left[1+\exp\left(\frac{V+60}{5}\right)\right]} \qquad \beta_{is} = \frac{1}{15\left[1+\exp\left(-\frac{V-18}{9}\right)\right]}$$

$$\tau_{if} = \frac{1}{\alpha_{if}+\beta_{if}} \qquad \tau_{is} = \frac{1}{\alpha_{is}+\beta_{is}}$$

Results

Kinetic properties of I_{to} in canine ventricular cells

I_{to} was activated by 300 ms long depolarizing voltage pulses arising from the holding potential of –80 mV to test poten-

tials gradually increasing up to +50 mV. Earlier results had suggested that I_{to} activates and inactivates so rapidly that it contributes only to the very early phase of repolarization. However, as illustrated in Figure 1A–D, inactivation of I_{to} could be fitted as a sum of two exponentials. The fast component had a time constant of less than 5 ms, which showed relatively little voltage-dependence. Following the rapid initial decay of current a second, much slower component of inactivation was also evident. Its time constant varied between 14 and 23 ms, while its amplitude reached 10–20% of peak current as a function of the membrane potential. This can result in a magnitude of outward current that represents a significant repolarizing force during the initial 100 ms of the action potential plateau, that is, in a critical period when other membrane currents are relatively week. Specifically, the magnitude of I_{to} was around 32 pA at 50 ms after the initiation of a voltage pulse to +30 mV, as calculated using the amplitude and inactivation time constant of the slow phase of I_{to}. For comparison, outward currents of approximately 15 pA and 10 pA were mediated by I_{Kr} and I_{Ks}, respectively, at the same time (see Varró *et al.*, 2000).

A steady-state activation relationship for I_{to} was obtained by applying a series of test pulses increasing up to +80 mV in 10 mV steps. The current peaks obtained were divided by the driving force at each membrane potential tested and these ratios were plotted against the respective test potential. When studying steady-state inactivation, test depolarizations were preceded by a set of 500 ms long prepulses clamped to various voltages between –90 and +20 mV. Peak currents measured after these prepulses were normalized to the peak current measured without prepulse (arising from the holding potential of –80 mV) and plotted against the respective prepulse potential. In both cases, data were fitted to the two-state Boltzmann function (Figure 1E–F). Closer inspection of the steady-state activation and inactivation curves obtained for I_{to} shows an overlap between the two curves revealing a 'window I_{to} current' similar to those reported earlier for I_{Na} and I_{Ca}. This steady-state current, which represents close to 5% of peak I_{to} at around –30 mV, may also contribute to repolarization during the late plateau or the early phase of terminal repolarization. In these experiments 1 μM nisoldipine, 0.1 μM dofetilide and 0.5 μM HMR 1556 were always present in the superfusate in order to completely eliminate I_{Ca}, I_{Kr} and I_{Ks}.

Based on data presented in Figure 1, significant lengthening of APD can be anticipated after suppression of I_{to}. In the absence of a selective blocker, this hypothesis cannot be tested definitively at present. However, the following results suggest that the established I_{Ks} blocker, chromanol 293B may be applied for this purpose.

Effect of chromanol 293B on potassium currents

A high concentration (100 μM) of chromanol 293B was shown to effectively block I_{Ks} without any effect on inward currents such as I_{Ca} and I_{Na} (Bosch *et al.*, 1998; Sun *et al.*, 2001). To study the effect of chromanol 293B on the various potassium currents governing ventricular repolarization in dogs, the compound was applied at a concentration of 100 μM. In these experiments 1 μM nisoldipine and 0.5 μM HMR 1556 were always added to the control superfusate in order to completely eliminate I_{Ca} and I_{Ks}. As shown in Figure 2A,B, 100 μM chromanol 293B markedly reduced the capacity of the I_{to}. At membrane potentials less than +20 mV block of the peak current was almost complete, while at more positive voltages it was only partial. However, chromanol 293B at the same concentration (100 μM) failed to significantly alter I_{Kr}, determined as tail current amplitude at –40 mV; this was not dependent on the membrane potential used to activate the current for 1 s (Figure 2C). Similarly, 100 μM chromanol 293B did not change the steady-state current–voltage relationship of the membrane, determined at the end of 300 ms long pulses clamped to potentials ranging from –80 to +30 mV; this overlaps the voltage range relevant to ventricular repolarization (Figure 2D). Within the negative region of this voltage range (between –80 and –30 mV) I_{K1} is active, while in the absence of I_{Ks} the positive branch of the I–V curve is dominated by a mixture of various non-inactivating potassium currents, often called plateau current (I_p). According to the results presented in Figure 2C,D, 100 μM chromanol 293B failed to modify any of the repolarizing currents (I_{K1}, I_{Kr}, I_p) other than I_{to} in the presence of HMR 1556.

Profile of I_{to} during the action potential

The most relevant way to estimate the profile of any current throughout the time course of the action potential is to apply action potential voltage clamp. This approach, however, requires the use of a selective blocker of the current. According to data in the literature (Bosch *et al.*, 1998; Sun *et al.*, 2001) and results shown in Figure 2, 100 μM chromanol 293B seems to be a selective blocker of I_{to}, provided the experiment is performed in the continuous presence of an I_{Ks} blocker (0.5 μM HMR 1556). The results of such an experiment are presented in Figure 3, and demonstrate the appearance of a small, persistent current component following the peak. The effect of chromanol 293B was largely reversible.

To avoid the distorting consequences of I_{Ca} rundown on the small slow component of I_{to} in conventional voltage clamp experiments an action potential waveform, which was previously recorded from a dog left ventricular epicardial slice with sharp microelectrode by conventional microelectrode technique, was delivered as the command potential. In this case any interference by other ion currents, including I_{Ks}, I_{Kr} and I_{Ca}, was removed by pharmacologically blocking these currents prior to the measurement. I_{to} was obtained by subtracting the current trace obtained in the presence of 100 μM chromanol 293B from the pre-drug current (Figure 4). The resulting chromanol-sensitive current, considered to be I_{to}, approximated the current profile expected to be generated during an action potential. At the beginning and during the late plateau this current was consistent with the slowly inactivating phase of I_{to}, which was previously demonstrated in Figure 1, using conventional square-pulse protocols and with the 'window-like' or 'late' components of the current.

Effect of chromanol 293B on action potential configuration

The consequences of I_{to} blockade on action potential configuration were studied in canine right ventricular subepicardial muscle preparations by applying 100 μM chromanol 293B in

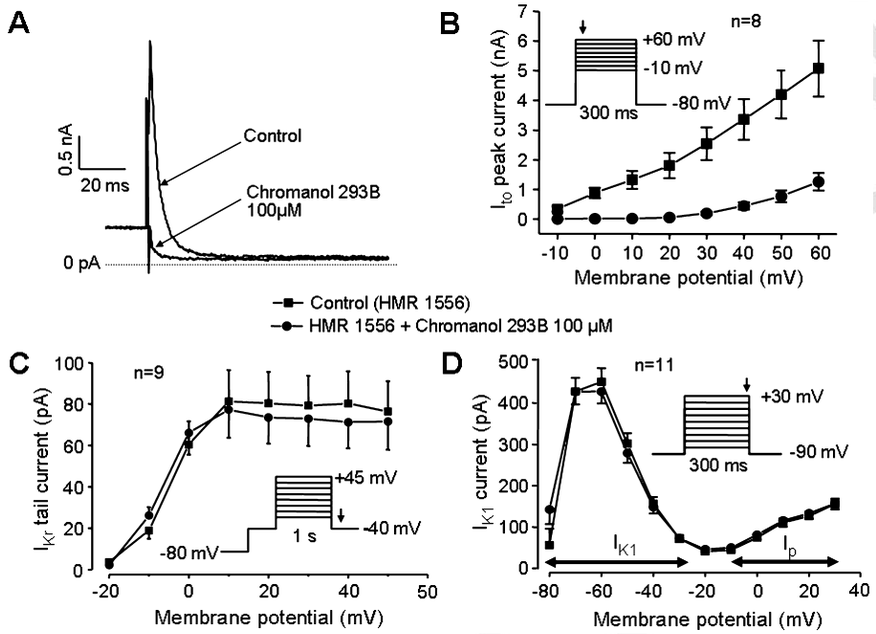

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Figure 2

Effects of 100 μM chromanol 293B on I_{to} (A, B), on I_{Kr} tail current (C), and on the steady-state current–voltage relationship obtained at 300 ms (D) in canine ventricular myocytes. The pulse protocols applied are shown in insets. When determining the I–V curve or measuring I_{to} test pulses were delivered at cycle length of 3 s, in the case of I_{Kr} measurements the repetition time was 20 s. The 500 ms long prepulse to –40 mV was applied to establish the baseline current when measuring I_{Kr}. Data are expressed as mean ± SEM. I_{Kr}, rapid component of the delayed rectifier potassium current; I_{to}, transient outward potassium current; *n*, number of measurements.

the presence of 0.5 μM HMR 1556 or 0.1 μM L-735 821 – both drugs providing full I_{Ks} blockade. This latter intervention was necessary to rule out possible changes due to the known I_{Ks} blocking property of chromanol 293B. As shown in Figure 5A, full inhibition of I_{Ks} caused only a slight lengthening of repolarization in subepicardial preparations, as was reported earlier in papillary muscles (Varró *et al.*, 2000). Additional suppression of I_{to} by administration of 100 μM chromanol 293B in the presence of I_{Ks} blockade significantly lengthened APD and decreased the amplitude of notch following early repolarization. These changes were accompanied by a marked positive shift of the plateau potential. The APD lengthening effect of I_{to} blockade showed inverse rate-dependent properties, in that it was more pronounced at slower than at faster pacing rates (Figure 5B).

Contribution of I_{to} to repolarization reserve

Repolarization reserve was greatly attenuated by application of 0.1 μM dofetilide for 60 min in the presence of I_{Ks} blockade. Dofetilide markedly lengthened the repolarization of right ventricular muscles by primarily delaying phases 2 and 3, without altering the notch (Figure 6A). This lengthening of repolarization – as expected – was inversely rate-dependent. After 40 min additional exposure to 100 μM chromanol 293B the prolongation of repolarization dramatically increased, especially at slow stimulation rates, also in an inversely rate-dependent manner. The magnitude of the chromanol-induced lengthening of APD at slow rates was greater than the arithmetical sum of the APD lengthening caused by chromanol 293B and dofetilide alone (Figure 6B). In addition, in five out of seven experiments early afterdepolarizations (EADs) were observed at cycle lengths longer than 2 s (Figure 6C). EADs were never observed under these experimental conditions when either HMR 1556, dofetilide or chromanol 293B were applied alone.

Computer simulations using action potential modelling

An updated dog epicardial action potential model was published recently by Decker *et al.* (2009). In this model, I_{to1} was formulated using a single exponential inactivation approach in the positive voltage range and without late 'window' type openings of the channels (no overlap between steady-state activation and inactivation curves). This formulation was carefully based on the available published data for dog ventricular muscle. Incorporating full I_{Ks} block in the model did not significantly change action potential configuration (see Figure 7A). Additional inhibition of I_{to} (90%) shifted the early plateau to more positive voltages and, in contrast to our experimental findings, shortened APD due to enhancement of I_{Kr} throughout the entire course of action potential (Figure 7B) and due to the accelerated inactivation of I_{CaL} compared with control conditions (Figure 7C). When I_{Kr} was partially inhibited (by 55 %) in the presence of full I_{Ks} blockade, lengthening of APD was observed. Additional inhibition of I_{to1} under these conditions resulted in a shortening of APD

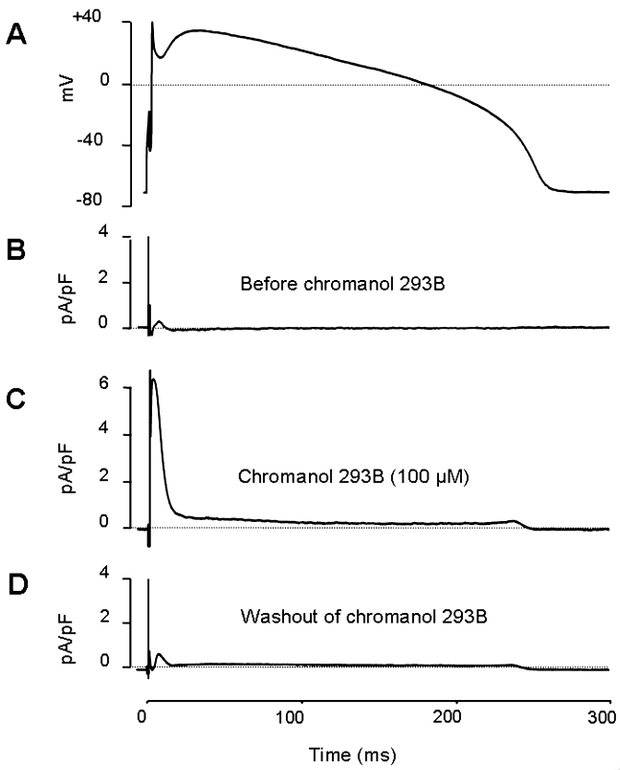

Figure 3

Records obtained from a myocyte under action potential voltage clamp conditions. In these experiments the action potential of each cell was used as command signal (A). After current traces were recorded under control conditions (B) 100 μM chromanol 293B was applied, which gave an inward compensatory current, the inversion of this current (C) was then defined as I_{to} (chromanol 293B sensitive current). (D) Shows a current trace recorded after removal of 100 μM chromanol 293B. I_{to}, transient outward potassium current.

(Figure 7D); this is not in line with the experimental results in this study.

However, when the formulation of I_{to} in the Decker model was changed to include the new results of this study shown in Figure 1 (the slow component of I_{to} inactivation and the late 'window' type channel openings due to the overlap of steady-state activation and inactivation curves), the simulations correlated well with the presented experimental data (Figure 8). As the simulation based on the modified model shows, inhibition of I_{to} in the presence of full I_{Ks} blockade produced a lengthening of APD (Figure 8A). In contrast to simulations obtained with the original model, there are three factors that co-operate to prolong repolarization at the mid-plateau area (indicated by a grey horizontal bar in Figure 8A) resulting in a concomitant lengthening of APD. These factors are: (i) a reduction in I_{Kr} during the initial 45 % period of the action potential in the case of I_{to} blockade, as shown in Figure 8B; (ii) an increased I_{CaL} (more inward current) following the rapid inactivation phase of I_{CaL} in the case of I_{to} blockade (Figure 8C, lower inset); and (iii) the late activation of I_{to} under control conditions due to the 'window' I_{to} current (Figure 8C, upper inset). Accordingly, 90% suppression of I_{to} after 100% I_{Ks} blockade plus 55% I_{Kr} blockade in the modified model yielded an excessive prolongation of repolarization resulting in the development of EADs (Figure 8D), further supporting the importance of I_{to} in repolarization when its late activation and slow phase of inactivation are taken into account.

Discussion and conclusions

The main finding and the message of the present study is that I_{to} is involved in governing repolarization by its so far not recognized kinetic properties in canine ventricular myocardium and, as a consequence, it contributes significantly to the repolarization reserve. Accordingly, inhibition of I_{to} in the presence of impaired repolarization reserve may elicit excessive repolarization lengthening resulting in EAD formation with the concomitantly enhanced risk of the development of pro-arrhythmias.

Our present experiments showed that the inactivation of I_{to} is a double exponential process, although there are many reports that have associated decay of I_{to} with a single exponential function (e.g. Wettwer *et al.*, 1994; Näbauer *et al.*, 1996; Akar *et al.*, 2004). The most likely reason that these studies found only a single component of inactivation for I_{to} is that the majority of these experiments were performed at room temperature. In addition, the second slow component can be easily overlooked in the nA scale as it is much smaller in amplitude than the fast one. Several studies, however, have reported a rapid and a slow phase of I_{to} inactivation at 36–37°C. The inactivation time constants found in our work are similar to those described in these studies (Han *et al.*, 2000; Zicha *et al.*, 2004). Our other important finding is that I_{to} shows a small overlap between the steady-state activation and inactivation curves revealing a 'window I_{to} current' similar to those reported earlier for I_{Na} and I_{Ca}. However, in several previous studies the existence of this 'window current' was not reported and the lack of effect of chromanol 293B on steady-state current in this 'window potential' range raises questions regarding the 'window I_{to} current' theory.

The controversy surrounding these results could be due to the unexplored and complicated nature of the gating mechanism of Kv4 channels, which are most likely the pore forming alpha subunit of dog I_{to}. Several studies have shown that the complexity of the gating of Kv4 channels is explained by models with multiple inactivation components accounting for both open- and closed-state inactivation. Upon reaching the open state, it is supposed that inactivation can proceed through either a closed-state mechanism or an open-state mechanism. The various gating models published in the literature agree on the predominance of closed-state inactivation in Kv4 channel gating (see review of Patel and Campbell, 2005). However, during the recovery period from open-state inactivation channels must reopen before reaching a closed state. On the basis of these models we can predict reopening of Kv4 channels upon membrane repolarization, which has been actually demonstrated with Kv4.2 channels (Gebauer *et al.*, 2004). This theory could be one possible explanation of the 'late' I_{to} current found in this study.

In our experiments we have shown that the slowly delaying phase of I_{to} and the 'late' component of the current may

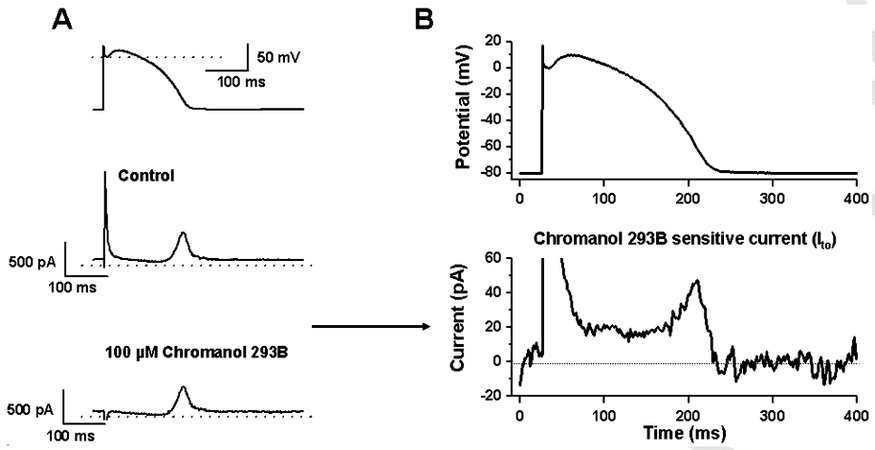

Figure 4

(A) I_{to} current profiles recorded under conventional voltage clamp conditions using a representative action potential as a command pulse. Other ion currents (I_{Ks}, I_{Kr} and I_{Ca}) were pharmacologically suppressed prior to the experiment. I_{to} was identified as a chromanol-sensitive difference current obtained by subtraction (B). I_{Ca}, L-type calcium current; I_{Kr}, rapid component of the delayed rectifier potassium current; I_{Ks}, slow component of the delayed rectifier potassium current; I_{to}, transient outward potassium current.

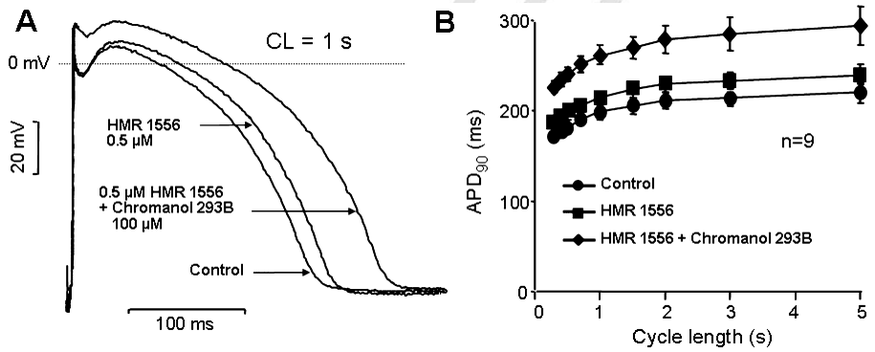

Figure 5

(A) Representative superimposed records demonstrating the effect of 100 µM chromanol 293B on action potential configuration when applied following pretreatment with 0.5 µM HMR 1556. (B) Cycle length-dependent changes in action potential duration (APD_{90}) measured under control conditions, following pretreatment with HMR 1556, and in the presence of HMR 1556 plus chromanol 293B. Symbols and bars denote mean ± SEM values obtained in nine multicellular right ventricular preparations. APD_{90}, action potential durations at 90% of repolarization.

carry a substantial current that then elevates the plateau voltage and delays both phase 2 and phase 3 repolarization.

The influence of I_{to} on cardiac action potential duration is not well understood in canine ventricle. In this preparation I_{to} has two components. One of these is Ca^{2+}-dependent and is carried by Cl^- (Zygmunt, 1994); however, the influence of this current on cardiac repolarization is hard to study, as intracellular Ca^{2+} dynamically changes during the action potential and is influenced by several factors. The other, calcium-independent K^+-mediated and strongly voltage-dependent component of I_{to} was more intensively studied, and revealed that it flows through Kv4.3 or Kv1.4 + KChiP2 channels. Kv1.4 has a slow recovery from inactivation, and therefore it can be expected to be largely inactivated at fast or normal heart rates. In the dog it is generally accepted that the main alpha subunit of the I_{to} channel is Kv4.3, which inactivates and recovers from inactivation relatively rapidly with time constants of approximately 10 and 50 ms, respectively (Patel and Campbell, 2005).

Therefore, I_{to} in the dog can influence cardiac repolarization within a wide range of heart rates. The general belief is that I_{to} contributes only to the early (phase 1) repolarization so it fails to directly influence APD, or it may alter APD indirectly by shifting the activation, deactivation or inactivation of other plateau currents. The impact of pharmacological block by 4-aminopyridine, the commonly used I_{to} inhibitor, on ventricular APD is controversial. Both moderate shortening and lengthening of APD have been reported with 4-aminopyridine – depending on the concentration, species, region of myocardium or experimental conditions (Litovsky and Antzelevitch, 1989; Kaab *et al.*, 1996; Zygmunt *et al.*, 1997; Greenstein *et al.*, 2000). The major problem with

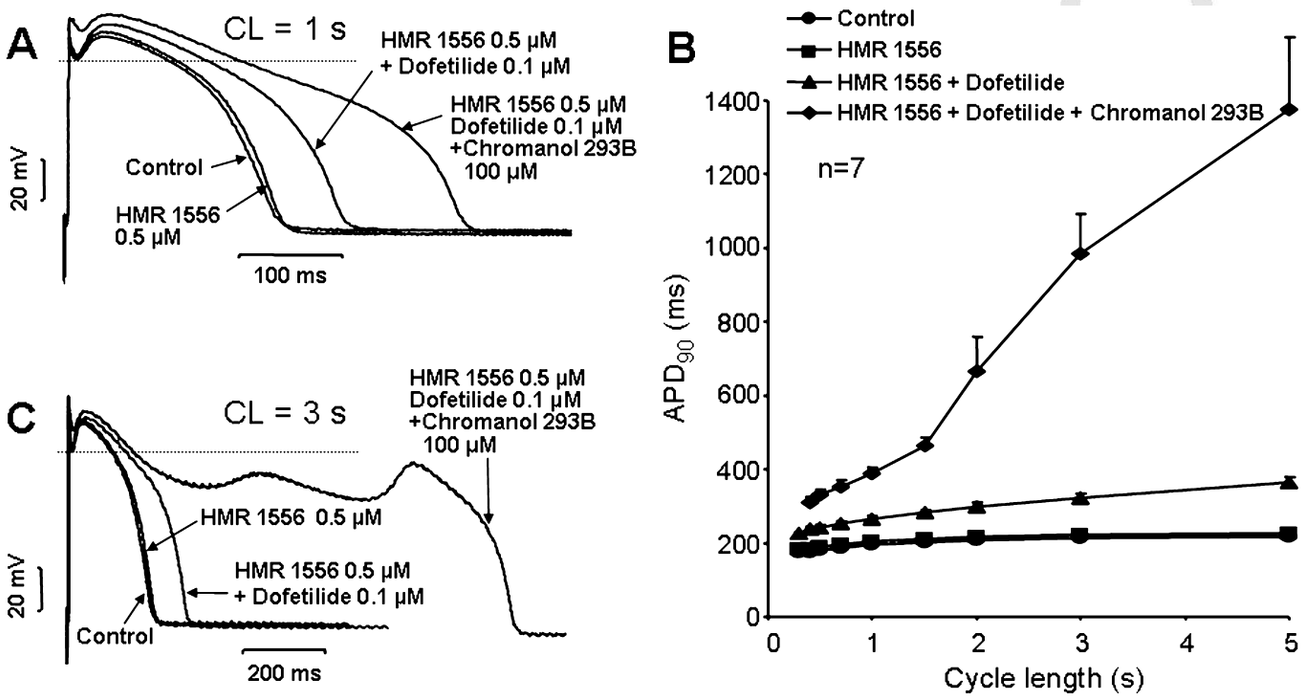

Figure 6

Contribution of I_{to} to repolarization reserve. (A, C) Representative superimposed action potentials recorded from multicellular right ventricular preparations at cycle lengths of 1 s and 3 s, respectively. In these experiments the preparations were cumulatively treated with HMR 1556, dofetilide and chromanol 293B (in the above sequence). HMR 1556 was used to prevent the I_{Ks} blocking effect of chromanol 293B, while dofetilide was applied to attenuate the repolarization reserve prior to chromanol 293B superfusion. (B) Cycle length-dependent changes in APD measured under the specified experimental conditions in seven multicellular right ventricular preparations. APD, action potential duration; I_{Ks}, slow component of the delayed rectifier potassium current; I_{to}, transient outward potassium current.

4-aminopyridine in action potential measurements is its lack of selectivity. Millimolar concentrations of 4-aminopyridine effectively suppressed I_{Kr} in addition to inhibiting I_{to} (Ridley *et al.*, 2003), and it has been suggested to have an inhibitory effect on I_{K1} (Van Bogaert and Snyders, 1982). Recently, canine ventricular myocytes were shown to express I_{Kur} current, and inhibition of I_{Kur} with low (submillimolar) concentrations of 4-aminopyride prolonged APD in these myocytes. These results greatly limit the value of 4-aminopyridine in action potential measurements indicating that data with 4-aminopyridine on cardiac repolarization should be interpreted with caution, and a more selective blocker of I_{to} (e.g. chromanol 293B in the presence of full I_{Ks} blockade) should be used to study the role of I_{to} in repolarization.

Indirect evidence supporting the conclusion of this study comes from the results of Hoppe *et al.*, who demonstrated that expression of I_{to} by gene transfer to guinea-pig ventricular myocytes, lacking endogeneous I_{to}, substantially shortened action potential duration (Hoppe *et al.*, 2000).

Lengthening of repolarization induced by 30 μM chromanol 293B in the presence of I_{Kr} blockade has been previously reported in dog ventricular muscle (Burashnikov and Antzelevitch, 2002) and this was attributed to the combined inhibition of I_{Ks} and I_{Kr}; however, the possible contribution of an I_{to} blockade was not considered. However, the present results suggest that this lengthening of repolarization could be attributed to the inhibition of I_{to}.

The new findings of this study incorporated into the existing dog action potential model regarding the kinetic properties of I_{to} resulted in a better match between the model-based predictions and experimental data, and as such represents an improvement of the existing knowledge and further suggest the importance of I_{to} in the overall cardiac repolarization.

Possible clinical significance

The clinical significance of alterations in I_{to} was previously recognized only in Brugada syndrome where it is thought that I_{to} contributes to the notch of the early repolarization phase of action potential (Sah *et al.*, 2003). The present study, although not focusing on this subject, points out the possibility of an enhanced risk for the development of pro-arrhythmias in cases of impairment, down-regulation or pharmacological suppression of I_{to}. The down-regulation of I_{to} has been well-demonstrated in heart failure (Beuckelmann *et al.*, 1993; Kaab *et al.*, 1996), in experimental diabetes (Magyar *et al.*, 1992; Shimoni *et al.*, 1994; Lengyel *et al.*, 2007), and in pathological conditions accompanied by an enhanced risk of sudden cardiac death (El-Atat *et al.*, 2004; Tomaselli and Zipes, 2004). Also, several commonly used drugs have been reported to inhibit I_{to} (Virág *et al.*, 1998; Iost *et al.*, 2003). More importantly, the majority of the drugs used in clinical practice have never been investigated with regard to their possible influence on I_{to}. Pharmacological investiga-

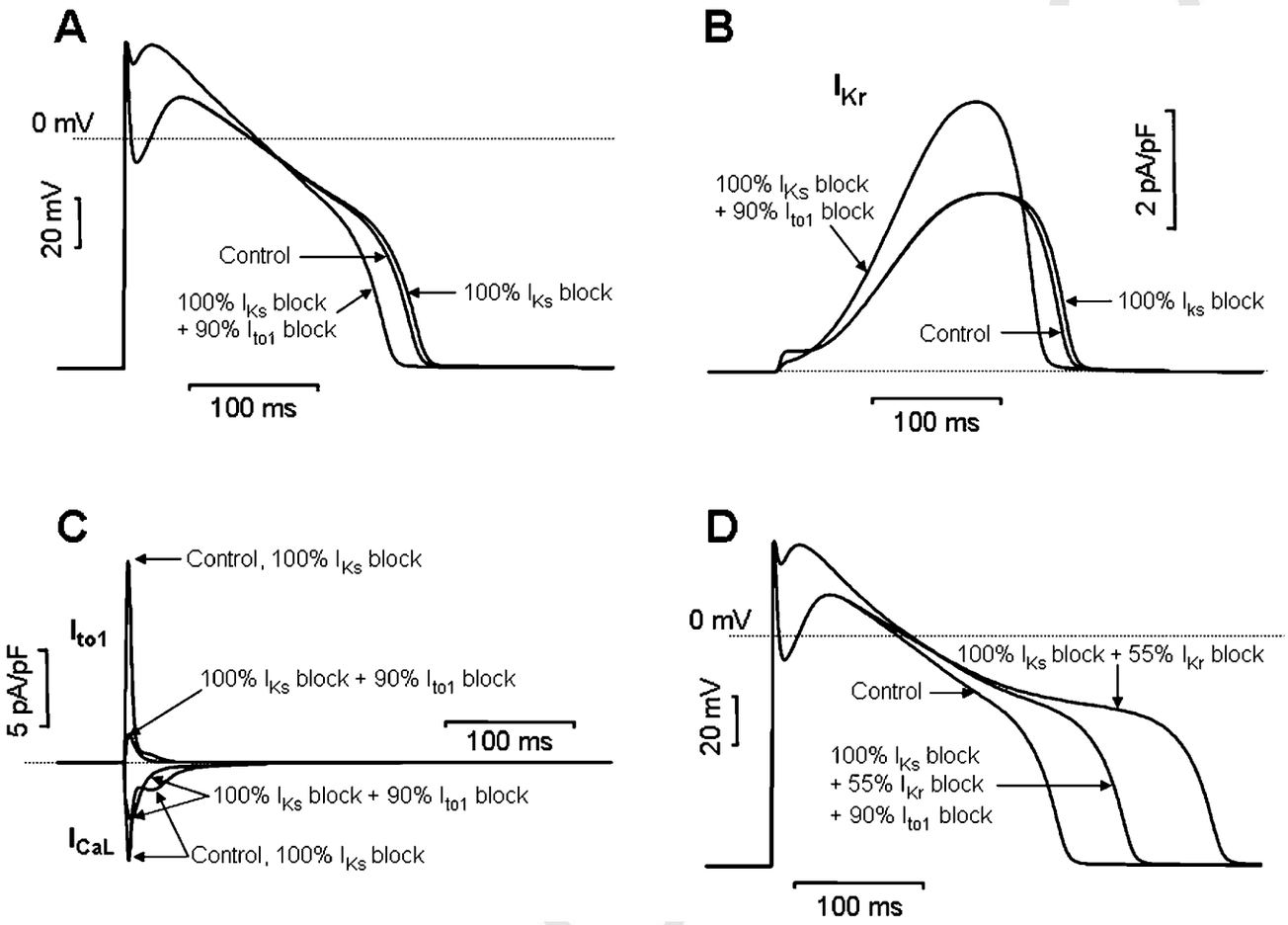

Figure 7

Simulated steady-state action potentials and the underlying ion current traces obtained using the original Decker *et al.* (2009) dog model. (A) Superimposed action potential records demonstrating the effect of 90% I_{to1} blockade on action potential configuration when applied together with a 100% block of I_{Ks}. (B) Superimposed I_{Kr} current traces under the same conditions as in (A). (C) Superimposed I_{to1} and I_{CaL} current traces under the same conditions as in (A). (D) Superimposed action potential records demonstrating the effect of 90% I_{to} blockade on action potential configuration in the presence of full I_{Ks} block plus partial (55%) blockade of I_{Kr}. I_{Ca}, L-type calcium current; I_{Kr}, rapid component of the delayed rectifier potassium current; I_{Ks}, slow component of the delayed rectifier potassium current; I_{to}, transient outward potassium current.

tions into the cardiac safety of drugs suggested by the authorities, normally emphasize only the importance of I_{Kr} (or herg current) measurements. Furthermore, these *in vitro* studies on APD, as well as *in vivo* studies on QTc duration of the ECG recordings are usually conducted in preparations having an intact repolarization reserve. In addition, the animals used for these investigations are often guinea-pigs or rabbits, species with cardiac preparations that do not exhibit or have a negligible I_{to} at their physiological heart rates.

Although some polymorphism in KCND2 and KCND3 genes has been found in hereditary LQTS patients, the incidence of these polymorphisms does not differ from the non-LQTS controls. Unfortunately, it was not demonstrated either how these mutations influence the function of Kv4.3 channels (Frank-Hansen *et al.*, 2005). It would therefore be interesting to consider the possibility of Kv4.3 and/or KChIP2 mutations in 'silent' or so far genetically negative LQTS patients, especially those who have had episodes or manifest LQTS after drug-treatment or in case of diseases such as hypothyroidism, heart failure or diabetes, that is, conditions where any further decrease in currents underlying repolarization may have particular significance.

Limitations of the study

Our conclusion was based on the assumption that chromanol 293B, in addition to blocking I_{Ks}, exerts its effect on cardiac repolarization by selectively inhibiting I_{to}. Although in the present study we carefully examined and ruled out the possible effects of chromanol 293B on other major transmembrane currents, such as I_{Kr}, I_{K1}, I_p, I_{Ca} and late I_{Na}, we cannot completely rule out the possible effects of chromanol 293B on all ion currents that may theoretically influence ventricular repolarization (e.g. calcium-dependent chloride current). It is also well known that there are significant transmural and regional differences in the expression of various transmembrane ionic currents, particularly of I_{to}. In our study we used

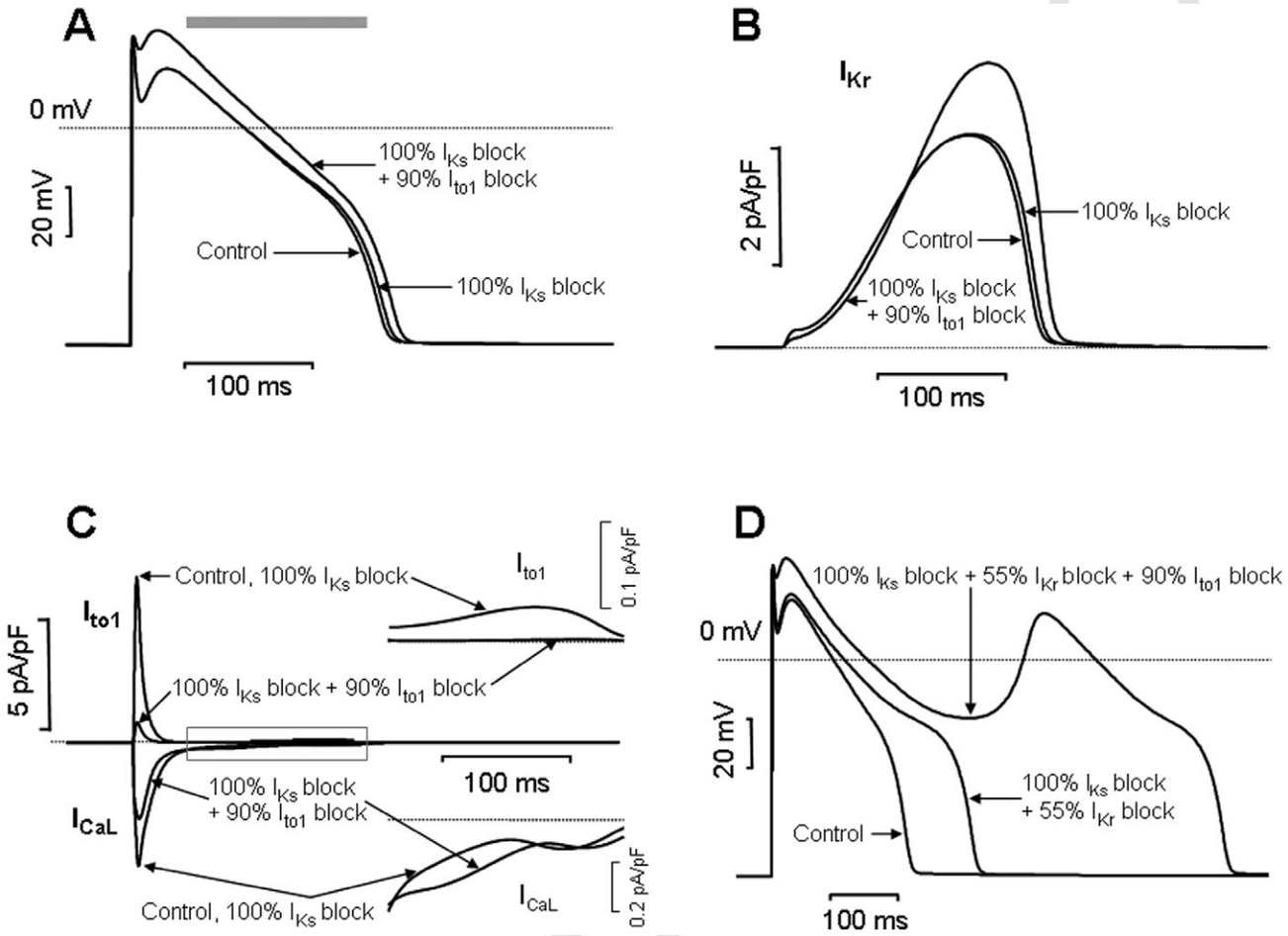

Figure 8

Simulated steady-state action potentials and ion currents obtained by the modified Decker *et al.* (2009) dog model. (A) Superimposed action potential records demonstrating the effect of 90% I_{to1} block on action potential configuration when applied together with a 100% block of I_{Ks}. The horizontal grey bar shows the time range in which ion currents were analysed. (B) Superimposed I_{Kr} current traces under the same conditions as in (A). (C) Superimposed I_{to1} and I_{CaL} current traces under the same conditions as in (A). The insets show both currents in details during the time-window specified by the grey rectangle in the main panel. (D) Superimposed action potential records demonstrating the effect of 90% I_{to} blockade on action potential configuration in the presence of full I_{Ks} block plus partial (55%) blockade of I_{Kr}. I_{Ca}, L-type calcium current; I_{Kr}, rapid component of the delayed rectifier potassium current; I_{Ks}, slow component of the delayed rectifier potassium current; I_{to}, transient outward potassium current.

right ventricular subepicardial muscle preparations to study the effect of I_{to} inhibition on repolarization, as this preparation was reported to strongly express I_{to}. Due to the existing transmural and regional inhomogeneity of I_{to} expression, inhibition of I_{to} may induce effects of different magnitudes in the various regions of the ventricular wall, which may further enhance the dispersion of repolarization, and as such is expected to increase the possibility of the development of pro-arrhythmias.

Conclusions

The main result of this study is that in the dog ventricular muscle, the voltage-dependent I_{to} has a second component with slow inactivation kinetics and late activation resulting in a window I_{to} current due to overlapping of the steady-state activation and inactivation curves. These direct effects of I_{to}, in addition to the previously recognized indirect effects on I_{Kr} and I_{Ca} caused by voltage changes, suggest that I_{to} makes a substantial contribution to repolarization during the plateau and terminal repolarization.

Thus, inhibition of I_{to} causes a positive shift of plateau voltage combined with a lengthening of the overall repolarization when studied in the presence of partially of fully compromised I_{Kr}. More importantly, the lengthening of APD under conditions of combined I_{Kr} and I_{to} blockade may be so excessive that it may result in the generation of EADs. As I_{to} is down-regulated in many diseases including heart failure, and because most of the drugs in clinical use have never been tested carefully for a possible inhibitory effect on I_{to}, the findings of the present study have important therapeutic and

safety implications regarding the risk of drug-induced QT prolongation and related life-threatening arrhythmias.

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Conflicts of interest

None.

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